Secreted substrates of Gram negative bacteria have to pass both the inner and the outer membrane. To accomplish this, these bacteria have developed different secretion mechanisms. To date at least six different types of secretion systems have been identified which are briefly discussed in **chapter 1** with an emphasis on their function, type of secreted molecules and their role in pathogenesis. Among these systems, the type IV secretion systems (T4SSs) are the most versatile and divergent in term of both prevalence and function. They are present in Gram negative and positive bacteria, in *Archaea,* and in wall-less bacteria [1]. T4SSs are ancestrally related to the plasmid conjugation systems, and still the majority of known T4SSs is plasmid encoded and responsible for conjugative transfer of DNA. Transfer of DNA via conjugation is one of the major causes of rapid horizontal gene transfer and leads to the rapid spread of antibiotic resistance. A prototype of conjugative T4SSs is encoded by the F plasmid of *E. coli* and has been described in great detail [2]. In *A. tumefaciens* DNA and effector molecules are transported via a T4SS resulting in crown gall tumors. The *vir* system of *A. tumefaciens* is the prototype of an effector translocator T4SSs, and has also been studied in great detail [3-4]. Over the last decades, increasing numbers of T4SS systems have been identified. Many of these systems have a similar organization and architecture as the *vir* system of *A. tumefaciens*. Several pathogenic bacteria utilize T4SSs to secrete virulent macromolecules that alter the host cellular response. Examples of these are the cag system of *H. pylori,* the ptl system of *B. pertussis* and the dot/icm of *L. pneumophila* that allow the bacteria to survive inside their host cells. Other T4SSs are adapted to divergent functions including DNA uptake from the medium (comB system of *H. pylori*) or the release of DNA into the medium (T4SS of *N. gonorrhoeae*). During the work described in this thesis the T4SSs of *Neisseria gonorrhoeae* were studied. *N. gonorrhoeae* is a Gram negative aerobic diploccocus. It is a human pathogen causing gonorrhea, the second most common sexually transmitted disease in the world. Although antibiotic therapy is still effective, the rapid rise of antibiotic resistance has resulted in strains that are increasingly resistant to the available treatments. The rapid spread of antibiotic resistance in gonoccoci is a consequence of highly efficient horizontal gene transfer resulting in a panmicitic gonoccocal population structure. *N. gonorrhoeae* encodes two types of T4SSs. A conjugative T4SS located on a gonococcal conjugative plasmid and a T4SS involved in DNA release encoded within the gonococcal genetic island (GGI). The functional and organizational characteristic of both systems are the subject of this thesis.

In **chapter 2,** the T4SS encoded within the gonoccocal genetic island (GGI) was studied. The GGI is a 57 kb chromosomally encoded genetic island inserted at the *dif* site in close proximity of the chromosomal origin of replication (*oriC*). A part of the GGI encodes a unique T4SS, which secretes DNA into the extracellular milieu. The secreted DNA is used for transformation of other gonococci, increasing the rate of DNA transfer approximately 500 fold [5]. In order to identify all the genes within the GGI, that are involved in DNA secretion, mutational studies were performed. Studies on the *exp1-parA* region showed that only the *parA* [5] and *parB* genes were essential for DNA secretion, whereas all the other genes in this region, could be deleted without any effect. Previous reports showed that two genes encoded by the first operon (*traD, traI*) are essential for DNA secretion [6-7]. Further mutational studies of the second operon showed that all genes with an exception for *traA, ybe, trbI, ybi, ycb* and *ych* were crucial for DNA secretion by gonococci [5, 8, this thesis]. Interestingly, the *traA* gene encodes the TraA pilin which is not essential for DNA secretion. Further analysis showed that the MS11A strain contains a frame shift mutation within the *traA* gene which results in a truncated form of the protein. Remarkably, the majority of gonoccocal strains contain a full length version of TraA. The effect of both *traA* forms on DNA secretion was tested. Surprisingly, the truncated version of *traA* does not affect DNA secretion while DNA secretion was inhibited by the presence of a full length protein. Mutations in TrbI, a leader peptidase-like serine protease, which catalyzes the circularization of the cyclic pilin subunit also led to defective pilin assembly and to DNA secretion. This demonstrated that substrate transport in T4SSs can occur in the absence of pilus. To examine the function of the full length version of TraA, conjugation assays were performed to test whether the T4SSs encoded by the GGI with a full length version of TraA mediate conjugal transfer of chromosomal markers, but no conjugation could be detected. Thus secretion of DNA is only found in a small subset of strains, which contain a mutation in TraA. Strains that do not contain the *traA* mutation do not secrete DNA, but also do not seem to be involved in conjugation.

The conjugative T4SS of *N. gonorrhoeae* was studied in **chapter 3.** Three types of gonoccocal conjugative plasmids have been described in *N. gonorrhoeae*; a 24.5 MDa plasmid with no detectable marker, and two 25.2 MDa plasmids which contain the *tetM* determinant [9]. Restriction endonuclease mapping and Southern blotting of conjugative plasmids from different isolates revealed two different 25.2 MDa *tetM* containing conjugative plasmids [10], which were named the "American" and "Dutch" type plasmid [11]. In chapter

3 tetracycline conjugative plasmids with a Dutch type backbone were identified in a large percentage of clinical isolates. The complete sequence of the Dutch type gonoccoccal tetracycline plasmid was determined. Within plasmids with a Dutch type backbone insertions of both Dutch and American *tetM* determinants were found. Next to the insertion of the different *tetM* determinants, the only difference between the strains with the *tetM* determinant (25.2 MDa plasmids) and strains without the *tetM* determinant (24.5 MDa plasmids) was the insertion of the *ngoSK11375* gene. The conjugative plasmids with the Dutch type backbone contained similarly to other IncP1 plasmids backbone modules for replication initiation, conjugative DNA transfer, mating-pair formation, stable plasmid inheritance and control. Genes encoded in the backbone modules of the Dutch type neisserial conjugative plasmids are phylogenetically divergent from the other IncP1 subfamilies, suggesting that they are members of a novel IncP1 subfamily. Interestingly, the Dutch type neisserial conjugative plasmids contain a 'genetic load', module not found in other IncP1 plasmids. Within this region three different putative toxin/antitoxin systems are located. Remarkably, two of the toxin/antitoxin systems belong to the Zeta/Epsilon toxin/antitoxin family. Therefore, Dutch type neisserial conjugative plasmids are a unique example of multiple copies of toxin/antitoxin systems. A third toxin/antitoxin system identified on the neisserial conjugative plasmid belongs to the VapD family. The *vapD* gene has been associated with virulence in several pathogenic bacteria [12-14] but its precise role is still unknown. In *H. influenzaea* the *vapX* gene was identified in the same operon as *vapD* and was shown to function as an antitoxin for VapD [15]. A homologue of the VapX protein was also found next to the *vapD* gene on the cryptic pJD1 plasmid of *N. gonorrhoeae* [16]. Remarkably, the Dutch type conjugative plasmid of *N. gonorrhoeae* contained only the *vapD* gene. Interestingly, the expression of the VapX antitoxin protein of pJD1 in *E. coli* acceptor cells strongly increased the transfer of the conjugative plasmid from *N. gonorrhoeae* to *E. coli*, indicating that the VapX protein located on pJD1 might function as an antitoxin for VapD on the conjugative plasmid. This might explain the limited host range of neisserial conjugative plasmids. Most plasmids contain only a single toxin/antitoxin system, and the advantage of having three different toxin/antitoxin systems on one conjugative plasmid remains unclear. Comparison of the T4SS system encoded by the conjugative plasmid and the T4SS encoded by the GGI showed that both systems function independently in transfer of only plasmid or only chromosomal markers, respectively.

The gonoccocal T4SS encoded within the GGI shows strong similarity with the protein sequences and the gene order of the F plasmid T4SS [5]. However, detailed comparison of both systems revealed several differences: i) the relaxase TraI of the GGI differs strongly from the relaxase of the F plasmid, ii) no accessory proteins for relaxosome formation like TraM or TraY have been found, iii) no cell to cell contact is required for activation of the DNA transport and iv) the pilin encoded in the GGI resembles the P-type pili found in *e.g.* the IncP incompatibility group and the *Agrobacterium tumefaciens* Ti plasmids. In **chapter 4** the F plasmid and the T4SS encoded within the GGI were compared in term of evolutionary relatedness and functional conservation. This demonstrated that the proteins of the mating pair formation complex were related to their homologs of the F plasmid, but that the proteins involved in targeting the DNA to the mating pair formation complex, the relaxase TraI and the coupling protein TraD cluster with proteins normally found in conjugative genetic islands, like the PAGI-2 and pKLC102 genomic islands of *Pseudomonas aeruginosa* and the PFGI-1 genetic island of P. *fluorescens Pf-5*. This suggests that the type IV secretion system of the GGI is composed of an F plasmid like mating pair formation system, but that the targeting components resemble conjugative systems found in genetic islands. Indeed it was previously shown that the gonoccocal TraI belongs to a novel family of relaxases [7]. One of the features of this novel family of relaxases is the presence of a domain of unknown function (DUF1528). In this study we tested whether gonoccocal TraD and TraI may substitute the function of the TraD and TraI deficient mutants of the F plasmid. Both proteins failed to interact with the F plasmid self-transfer functions and for mobilization of the *oriT* plasmid. That again showed that either both the F plasmid and the GGI function in a different manner or more gonococcal components, possibly the coupling protein and the relaxase together need to be provided *in trans* to complement the deletions.

T4SSs play a crucial role in bacterial pathogenesis. In the past few years remarkable progress has been made in identifying T4SSs of bacterial pathogens and in the understanding of their roles and substrates in pathogenesis. However, many aspects remain to be still investigated. Data presented in this thesis pose new challenges for future. One of the main goals will be to understand the functional importance of the presence of a non mutated TraA in neisserial pathogenesis. Secondly, the completed sequence of the neisserial conjugative plasmids will give more opportunities to study this T4SS system in more details.